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A Study of the Degradation *in vitro* of Organochlorine Compounds by the Meat Starter Micro-organism *Micrococcus varians*

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Introduction

The extensive use of chlorinated pesticides (OCPs), as well as of other chemicals such as polychlorinated biphenyls (PCBs), has led to the contamination of the environment and of foodstuffs, especially those having a high fat content. These man-made chemicals are halogenated, and highly persistent.

The intention of the regulatory agencies and food manufacturers is to minimize the exposure of the public to these potentially hazardous compounds whenever possible. This philosophy prompts active research into the extent to which organochlorine residues can be removed during technological operations. Many studies on pesticide and PCB residues have revealed that certain factors significantly reduce the levels of such compounds in the course of food processing. These residues can be partially or wholly removed or degraded to different derivatives by mechanical, thermal, chemical or biochemical agents.[1]

Biodegradation of chemicals by living organisms is one of the most important mechanisms for the breakdown of organic compounds and micro-organisms are the most important agents for such degradation. However, degradation is a very specific process and the growth of some micro-organisms can even be inhibited by a xenobiotic. If degradation does occur, it is likely to result from enzymatic activity and may either occur immediately or only after a period of adaptation to the chemical.[2]

Reports on microbial degradation of OCPs and PCBs appear in increasing numbers, but such investigation tend to be focused on soil or aquatic micro-organisms,[3–7] while the activity of micro-organisms associated with food fermentation has been less well investigated.[8–11]

Residues of organochlorine pesticides and polychlorinated biphenyls have been found in meat and meat products,[12–18] and the study of degradation of such residues in these foodstuffs is very important because of their increasing rate of consumption worldwide. Nowadays, in the meat industry it is very common to use starter cultures to improve the characteristics of the meat products, and the possibility that these micro-organisms would degrade these contaminants is of great interest because the dechlorinated products are generally less toxic to animals, less likely to bioaccumulate, and more susceptible to further microbial attack. In order to investigate this, some in-vitro studies on the degradative activity of *Micrococcus varians* (a microbial isolate from a commercially available meat starter culture) on PCBs and OCPs in culture media were performed. The technique of capillary gas-liquid chromatography (GLC-ECD) was used to quantify the modifications of the different compounds. Simultaneously, the growth pattern of *M. varians* in the

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culture medium containing the organochlorine compounds was investigated in order to observe any inhibitory effect on growth that could inhibit its degradation capabilities.

Materials and methods

All test compounds were of >99.0% purity. Reagents were HPLC grade or better and all apparatus was cleaned thoroughly, dried and then rinsed with hexane before use. All solvents were checked for the absence of components which would produce interfering peaks in the GC analysis.

Materials. Water was distilled using a MilliQ water purification system and extracted with hexane prior to preparation of the liquid media.

Tryptic soy broth (TSB; Difco) contained (g litre^{-1}): tryptone (pancreatic digest of casein) (17); soytone (papaic digest of soy bean meal) (3); dextrose (2·5); sodium chloride (5) and dipotassium phosphate (2·5).

The mineral salts medium (MSM) contained (g litre^{-1}): Na_2HPO_4 (7); KH_2PO_4 (3); NaCl (0·5); NH_4Cl (1); $MgSO_4.7H_2O$ (0·25) and yeast extract (Difco) (1).

Tryptic soy agar (Difco) contained (g litre^{-1}): tryptone (pancreatic digest of casein) (15); soytone (papaic digest of soy bean meal) (5); sodium chloride (5) and agar (15).

For dilutions, aqueous peptone (Difco; 1 g litre^{-1}) was used.

Bacterial strain. Micrococcus varians was isolated from the commercially available meat starter culture SAGA L® (manufactured by Quest International and supplied by Amerex Laboratories, Madrid, Spain). The strain was stored in skim-milk vials at −18°C until utilized. An 18-hour-old actively growing culture in broth was used to inoculate the sterile media, to give a final concentration of 10^5 cells ml^{-1}.

Degradation study. Tubes containing 9·9 ml of the sterilized liquid medium inoculated with *M. varians*, were spiked with 0·1 ml of a standard OCP/PCB mixture in acetone in which each compound was present at 100 μg ml^{-1}. Uninoculated samples containing the organochlorine compounds were used as controls. Tubes were incubated at 30°C on a rotary shaker for seven days.

After incubation, the residues of OCPs and PCB 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) remaining in the medium were extracted following the general method described by Viney and Bewley[19] with slight modifications: the medium (10 ml) was shaken with hexane (40 ml; 1 h) and the liquid then transferred to a separatory funnel, using hexane (5 ml) as a rinse. The organic layer was removed, the separatory funnel rinsed with hexane (5 ml) and the volume reduced to 10 ml in a rotary evaporator before it was transferred to a vial. This solution was diluted 10-fold with hexane before analysis. Each *in-vitro* assay was performed in triplicate.

Analysis of degradation products. Chromatographic analyses were performed with a Hewlett-Packard HP 5890 system with ^{63}Ni electron capture detector (ECD), equipped with an automatic injector HP 7673A. A fused silica capillary column coated with 5% phenyl methyl polysiloxane (Quadrex 007-2, New Haven, CT 50 m × 0·25 mm ID × 0·25 μm film thickness) was used. Data acquisition and processing were performed on a HP Vectra 486/33U computer using Hewlett-Packard Chemstation software.

The chromatograms of the extracts from the inoculated and uninoculated samples were compared to determine whether the organism had altered the residual amount of the organochloride compounds in the medium.

For every *in-vitro* assay, a procedural blank consisting of all reagents and glassware used during analysis was run to check for intereferences and cross-contamination. Full details of the methods are in Ref. 20.

Analytical precision. Recoveries of organochlorine pesticides and PCB 153 by this method were determined by fortification of the two liquid media with an acetone solution of the investigated compounds at the same concentration as that used in the experiments, and ranged from 80 to 110%, in agreement with FDA recommendations.[21] The repeatability was very good with relative standard deviations in the range 2·5–4·8%.[20]

Influence of the mixture of OCPs and PCB 153 on the growth of Micrococcus varians. Tubes containing liquid medium (9·9 ml) inoculated with *Micrococcus varians* were spiked with the standard solution described above (0·1 ml). Other tubes of the same liquid medium received acetone only. Each treatment was performed in duplicate and incubated at 30°C on a rotary shaker for seven days. Growth in both media was determined by plate counts in tryptic soy agar (TSA) after 0, 1, 2, 5 and 7 days' incubation. The results were compared with those for *M. varians* in TSB and MSM without organochlorine residues or acetone.

Measurement of pH of the media at the initial stage and at the end of the incubation period was performed with a pHmeter (Crison).

Results

Degradation of OCPs and PCB 153 by Micrococcus varians. In the nutrient medium TSB, there was a slight reduction of hexachlorobenzene (HCB), dieldrin and the isomers α- and γ- of hexachlorocyclohexane (1·0–2·2%), but considerable reduction of *pp*′DDE and PCB 153 (5·9 and 7·5% respectively). β-Hexachlorocyclohexane (HCH) content was not noticeably reduced (Table 1).

M. varians was able to degrade some of the highly persistent compounds tested in the mineral salt

medium. Significant reductions ($P < 0{\cdot}05$ by Mann-Whitney's nonparametric test) of HCB (12·7%), *pp*′DDE (17·7%), dieldrin (16·7%) and PCB 153 (15·5%) occurred in this medium, whereas the HCH isomers content was practically unaltered (Table 1).

Effect of OCPs and PCB 153 on growth of Micrococcus varians. In this investigation, the term 'microbial growth' is related to the multiplication of the microbial population, which might be affected by the addition of the zenobiotic. The *M. varians* counts after incubation in medium containing acetone or the mixture of xenobiotics are shown in Table 2.

Addition of 0·1 ml of acetone did not affect growth of *M. varians* in either medium. The addition of the mixture of OCPs and PCB 153 resulted in decreases in counts of the bacterium during the initial 24 h of incubation in MSM. Then, the micro-organism recovered and began to grow logarithmically, but not as well as in a normal situation. Viable counts in TSB spiked with the organochlorine compounds were below those for growth in medium in the absence of added compounds.

Initial values of pH were 7·2 in TSB and 7·1 in MSM. At the end of incubation time, values of pH ranged from 6·0 to 6·1 in TSB and from 6·9 to 7·0 in MSM (Table 2).

TABLE 1
Reduction of the Organochlorine Pesticide and Polychlorinated Biphenyl Contents in Liquid Media Incubated with *Micrococcus varians*

	Tryptic soy broth	*Mineral salt medium*
Compound	*Reduction* (%)[a]	
HCB	2·1	12·7[b]
α-HCH	1·0	1·2
β-HCH	0	0
γ-HCH	1·2	0·8
pp′DDE	5·9	17·7[b]
Dieldrin	2·2	16·7[b]
PCB 153	7·5	15·5[b]

[a] Mean percentage from three *in-vitro* assays.
[b] Significant reduction ($P < 0{\cdot}05$; Mann-Whitney's nonparametric test.

Discussion

The different degradation results obtained in the two media were not surprising since the microbial metabolic activity can be influenced by the composition of the culture medium. This difference in breakdown pattern could take place in a real situation *in vivo*, particularly in food products with different nutrient content. Degradation did not take place in nutrient broth, possibly because *M. varians* preferred to use other organic substrates than the pesticide, or the xenobiotic may have formed a complex with some components of the medium, avoiding the degradative activity of microorganisms, as demonstrated by Langlois[22] and Langlois *et al.*[23] It would be of great interest to investigate the effect of inoculating *M. varians* in meat for the manufacture of dry sausages, to compare our in-vitro results validly with the situation *in vivo* in foodstuffs, in the presence of nutrients, but with different medium and different growth conditions.

In other *in-vitro* studies with microbial strains relevant to the meat industry, Peric *et al.*[11] observed that diverse micrococci strains, isolated from fermented sausages, could degrade α-, β-, γ-HCH, *pp*′DDT and methoxychlor after 48 h at 37°C. Average percentage reductions were higher than the reductions for HCH isomers in our study: 28% for α-HCH, 37% for β-HCH, 48% for γ-HCH or lindane, 43% for *pp*′DDT and 59% for methoxychlor. Spiric *et al.*[24,25] isolated diverse micrococci strains that could significantly degrade DDT and lindane in a nutrient medium, and Mirna and

TABLE 2
Viable Counts of *Micrococcus varians* in Tryptic Soy Broth and Mineral Salt Medium as affected by the Addition of Organochlorine Contaminants or Acetone

	Counts (ml^{-1}) *in tryptic soy broth*			*Counts* (ml^{-1}) *in mineral salt medium*		
Time (*h*)	*A*[a]	*B*[a]	*C*[a]	*A*[a]	*B*[a]	*C*[a]
0	$5{\cdot}0 \times 10^5$	$5{\cdot}0 \times 10^5$	$5{\cdot}0 \times 10^5$	$6{\cdot}5 \times 10^5$	$6{\cdot}5 \times 10^5$	$6{\cdot}5 \times 10^5$
24	$5{\cdot}1 \times 10^8$	$4{\cdot}1 \times 10^8$	$2{\cdot}2 \times 10^7$	$1{\cdot}4 \times 10^8$	$2{\cdot}6 \times 10^8$	$3{\cdot}3 \times 10^5$
48	$1{\cdot}1 \times 10^9$	$8{\cdot}4 \times 10^8$	$1{\cdot}1 \times 10^8$	$1{\cdot}5 \times 10^8$	$2{\cdot}1 \times 10^8$	$9{\cdot}5 \times 10^6$
120	$8{\cdot}4 \times 10^8$	$9{\cdot}2 \times 10^8$	$1{\cdot}0 \times 10^8$	$1{\cdot}3 \times 10^8$	$2{\cdot}1 \times 10^8$	$3{\cdot}2 \times 10^7$
168	$8{\cdot}3 \times 10^8$	$6{\cdot}7 \times 10^8$	$9{\cdot}8 \times 10^7$	$1{\cdot}6 \times 10^8$	$1{\cdot}5 \times 10^8$	$3{\cdot}4 \times 10^7$
pH[b]	6·1	6·1	6·0	7·0	7·0	6·9

[a] A, without additives B; acetone (10 ml $litre^{-1}$) in the medium; C. OCP/PCB mixture in the medium.
[b] Final pH values in the medium; the initial pH of tryptic soy broth was 7·2 and of mineral salts medium 7·1.

Coretti[10] observed a reduction of between 20 and 30% of lindane, and a degradation of DDT in TSB (mainly to DDD) by the action of a micrococus strain from a commercial meat starter culture, while the strain of *M. varians* investigated in the present study had no relevant degradative effect in the presence of nutrients.

Furukawa *et al.*[26] stated that the capacity to degrade PCBs varies between bacteria, and that some bacteria are capable of degrading highly chlorinated compounds. Viney and Bewley[19] demonstrated degradation of PCB 153 by soil micro-organisms similar to that found in our work.

The degradative activity of *M. varians* could be of great value for detoxifying the widespread organochlorine contamination that occurs mainly in foods of animal origin, like meat and meat products. In relation to this, investigations made in our laboratory[13,14,17] demonstrated that sausage curing for one month caused a 30% reduction in naturally occurring lindane levels, whereas residues of α-hexachlorocyclohexane in meat decreased by almost 25%. These reductions were attributed to a possible microbial degradation by the fermented meat micro-flora. However, the HCB and *pp*′DDE contents were not significantly reduced after curing (9·5% reduction in HCB content, and the mean DDE level was nearly the same after curing). In the present study, the *in-vitro* results with *M. varians* were different from those obtained by Ariño *et al.*[13,14,17] in meat products. The reduction of HCB in mineral salts medium was greater than the 9·5% found after the processing of sausages, and the DDE content was significantly reduced, while α- and γ-HCH were not noticeably reduced by the strain. Extra care must be taken when comparing the two sets of results since *in-vitro* studies are not always relevant to the real situation in food products. This is due to the fact that the biodegradation process may be affected by a number of factors such as the type of micro-organism (even the type of strain), the interaction between micro-organisms, the microbial concentration, the nature of the xenobiotic and its concentration, the composition of the medium, whether the medium is liquid or solid, and the microbial growth conditions of temperature and pH.

Studies made by Kohler *et al.*[27] and Jagnow *et al.*[28] demonstrated that the physiological state of the bacteria influences the biodegradation process. As we have observed, the mixture of OCPs and PCB 153 had an initial bacteriostatic effect on *M. varians*, and this fact could affect its degradation activity both *in vitro* and in a real situation in meat products.

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